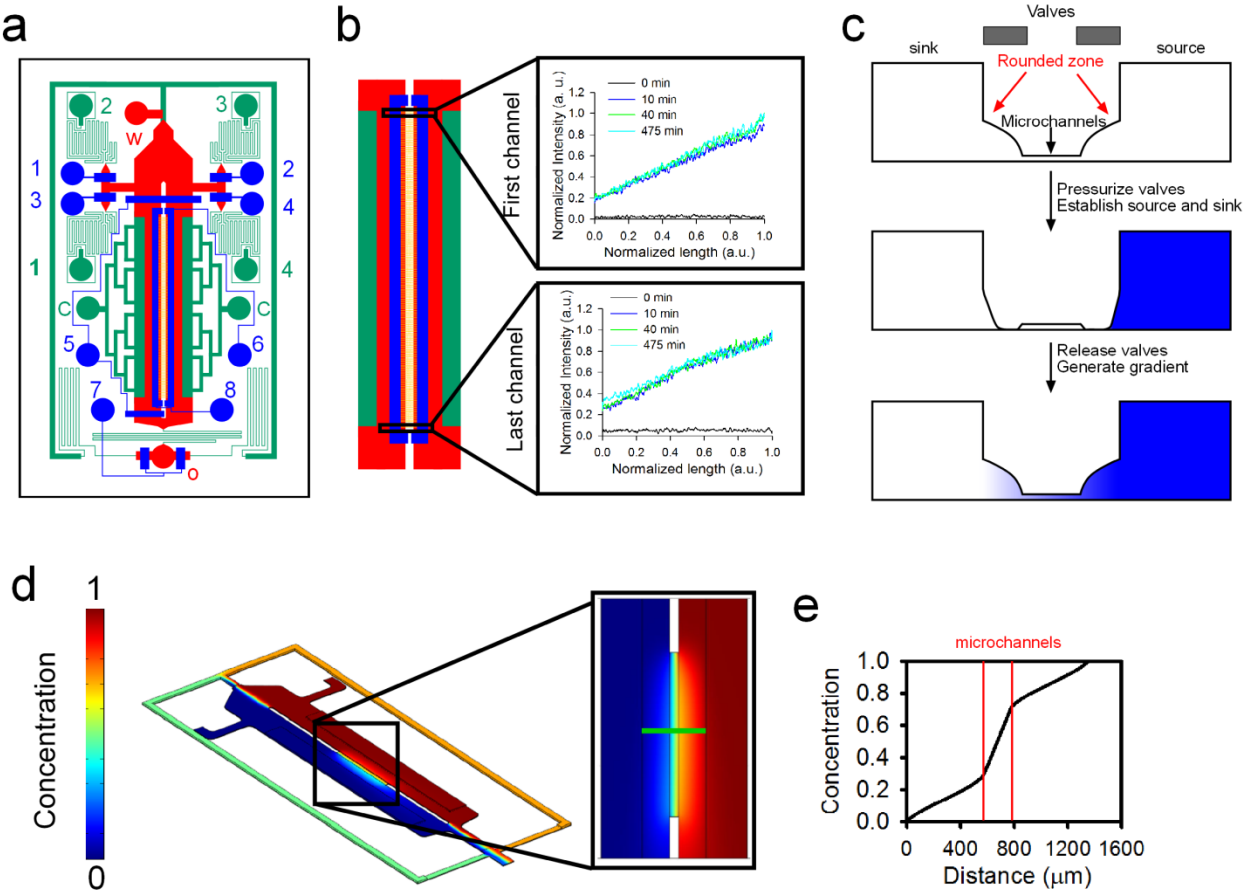
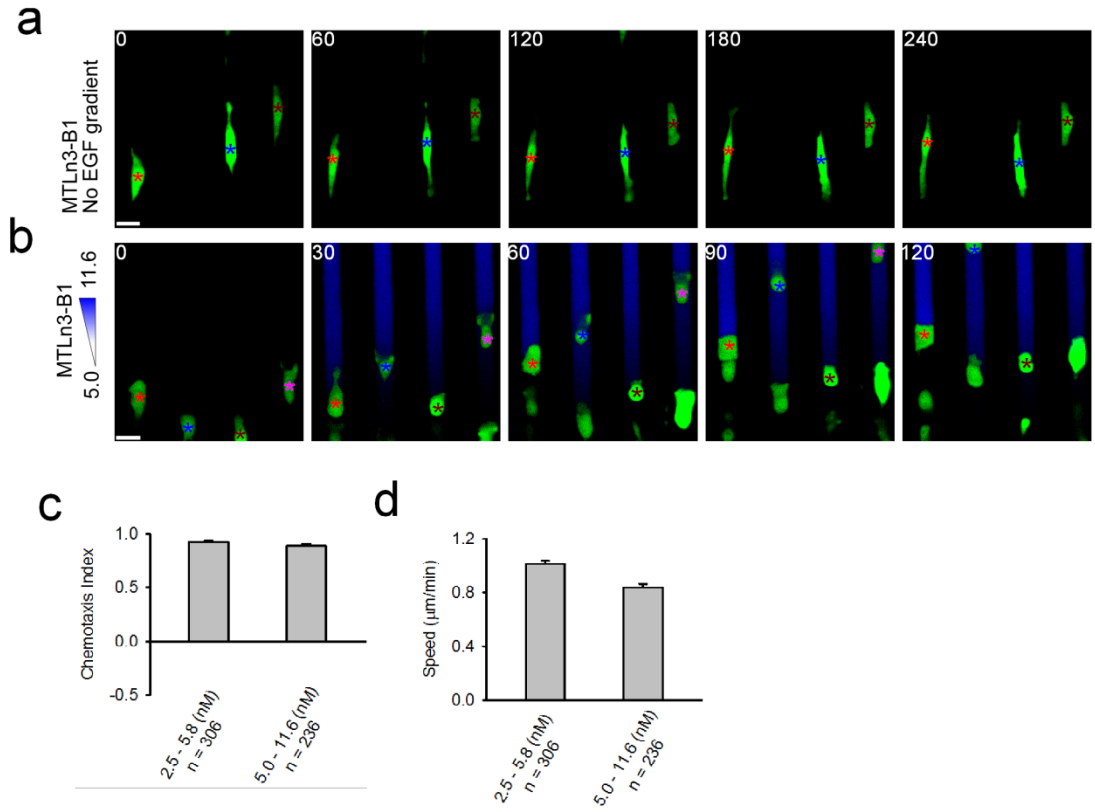
# Supplementary figure 1



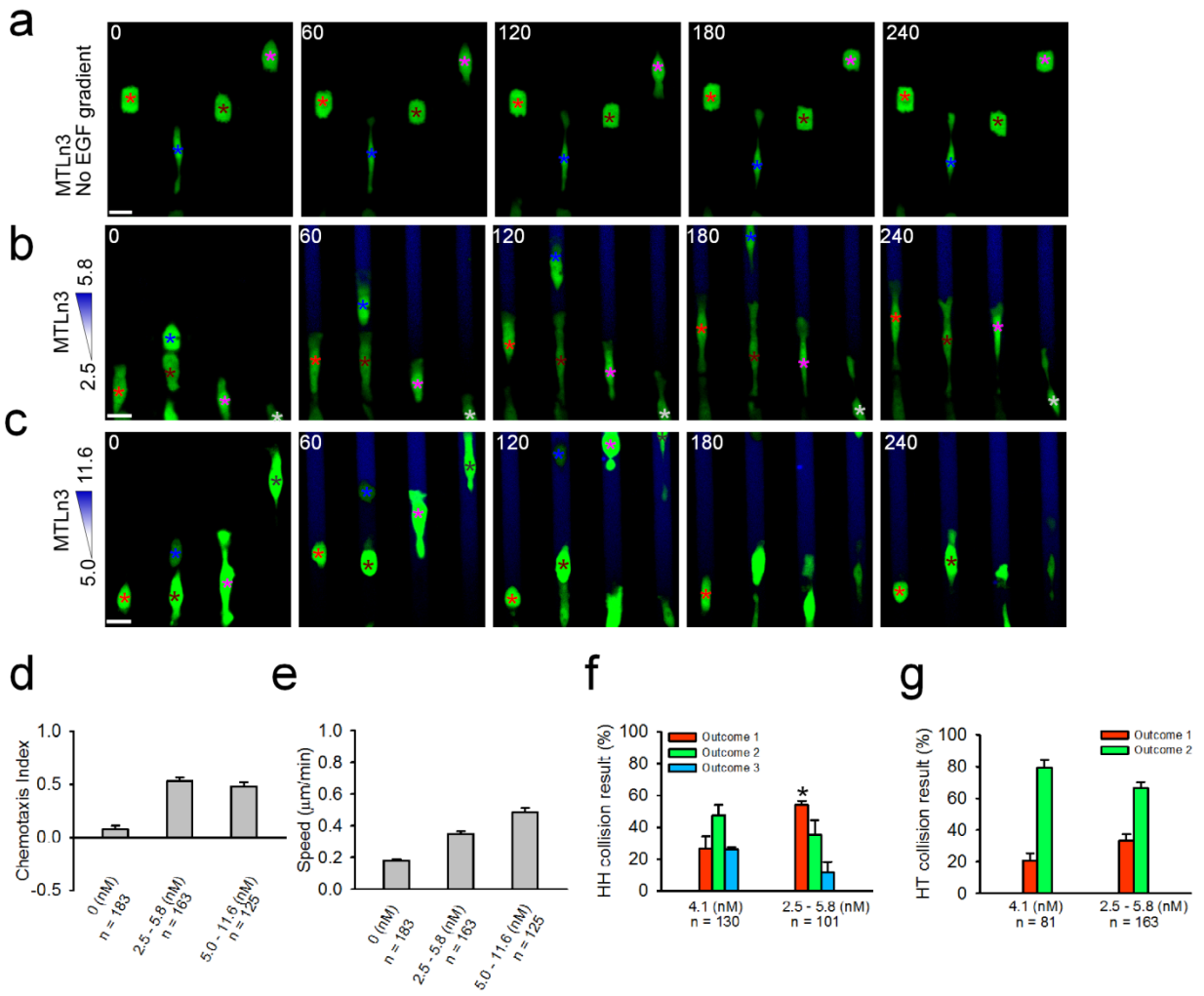

**Supplementary figure 1. Characterization of the microfluidic device used to study chemotaxis and CIL.** (**a**) Schematic of the microfluidic device. Different colors represent different functional layers. The green fluidic layer is 130 μm, the red rounded fluid layer is 30 μm, and the yellow microchannel layer is 6 μm. The blue layer is a valve layer (40 μm) that controls fluid flow in the underlying red rlayer. (**b**) Enlarged view of the functional gradient generation area and quantification of gradient formation kinetics in the first microchannelchannel and last microchannel channel. Gradient formation begins simultaneously across all channels, becomes stables in ~10 minutes, and is stable over the 8 hour experimental time period. (**c**) A side view illustrating the gradient formation strategy. (**d-e**) 3D computational fluid dynamics simulations of gradient generation in the microfluidic device. Quantification of the green line shown in the enlarged region is shown in (**e**). The concentration difference across the microchannels is 30-70%.

# Supplementary figure 2



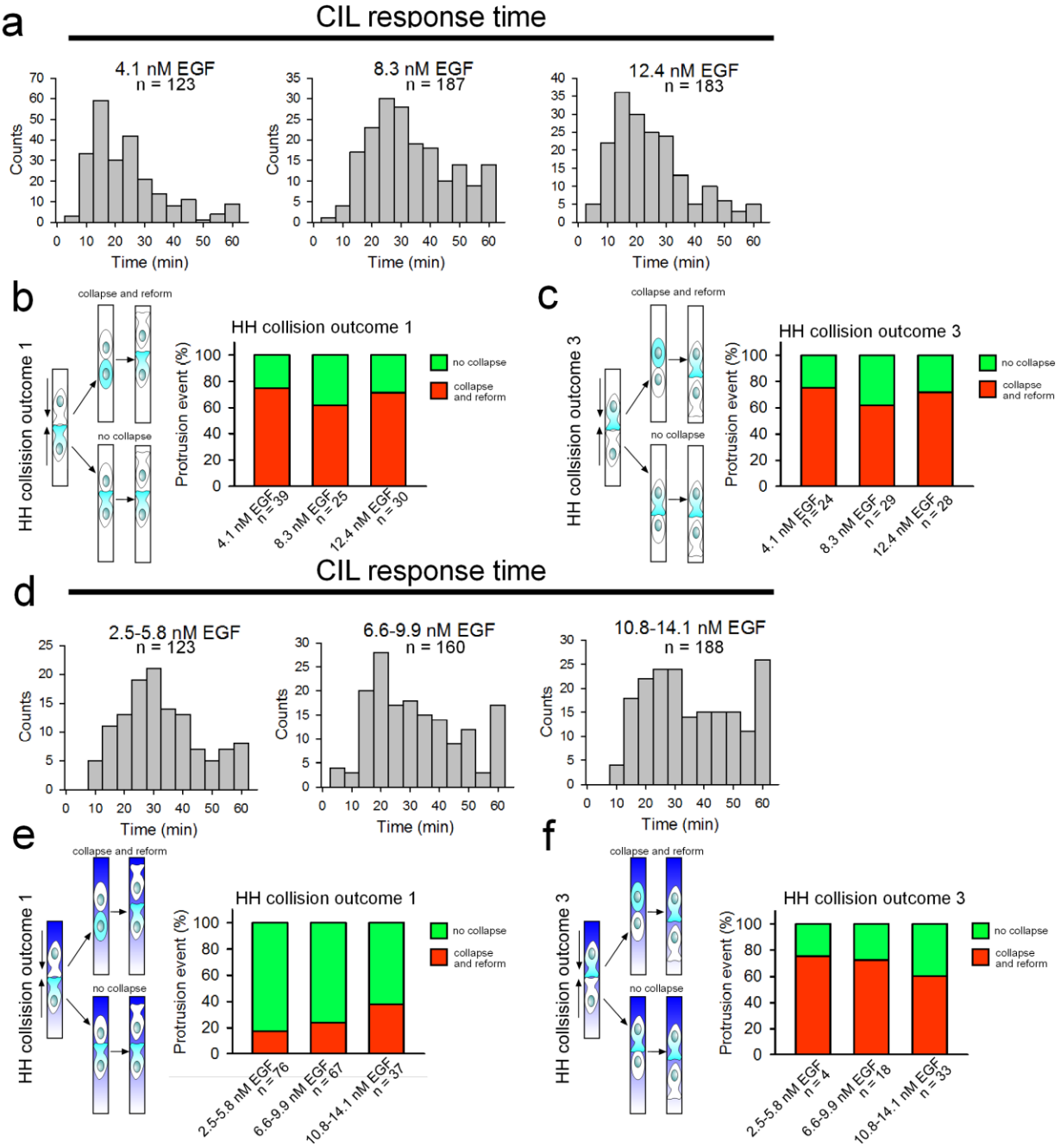

**Supplementary figure 2. MTLn3-B1 cells in control conditions and sharper EGF gradients.** (**a**) MTLn3-B1 cells in microchannels without EGF. (**b**) MTLn3-B1 cells in 5.0-11.6 nM EGF gradients. In **a-b,** green color denotes GFP expression, the blue color visualizes the gradient of EGF using dextran dye as a proxy, and different colored asterisks track individual cell positions. Times are in minutes. Scale bars, 20 μm. (**c-d**) Quantification of the chemotaxis index (**c**) and velocity (**d**) of MTLn3-B1 cells in 5.0-11.6 nM EGF gradients as compared to 2.5-5.8 nM EGF gradients. 2.5-5.8 nM EGF data is reproduced from Fig. 1c-d. Data represents the mean from the number of cells indicated from n = 3 independent experiments with error bars showing SEM.

# Supplementary figure 3



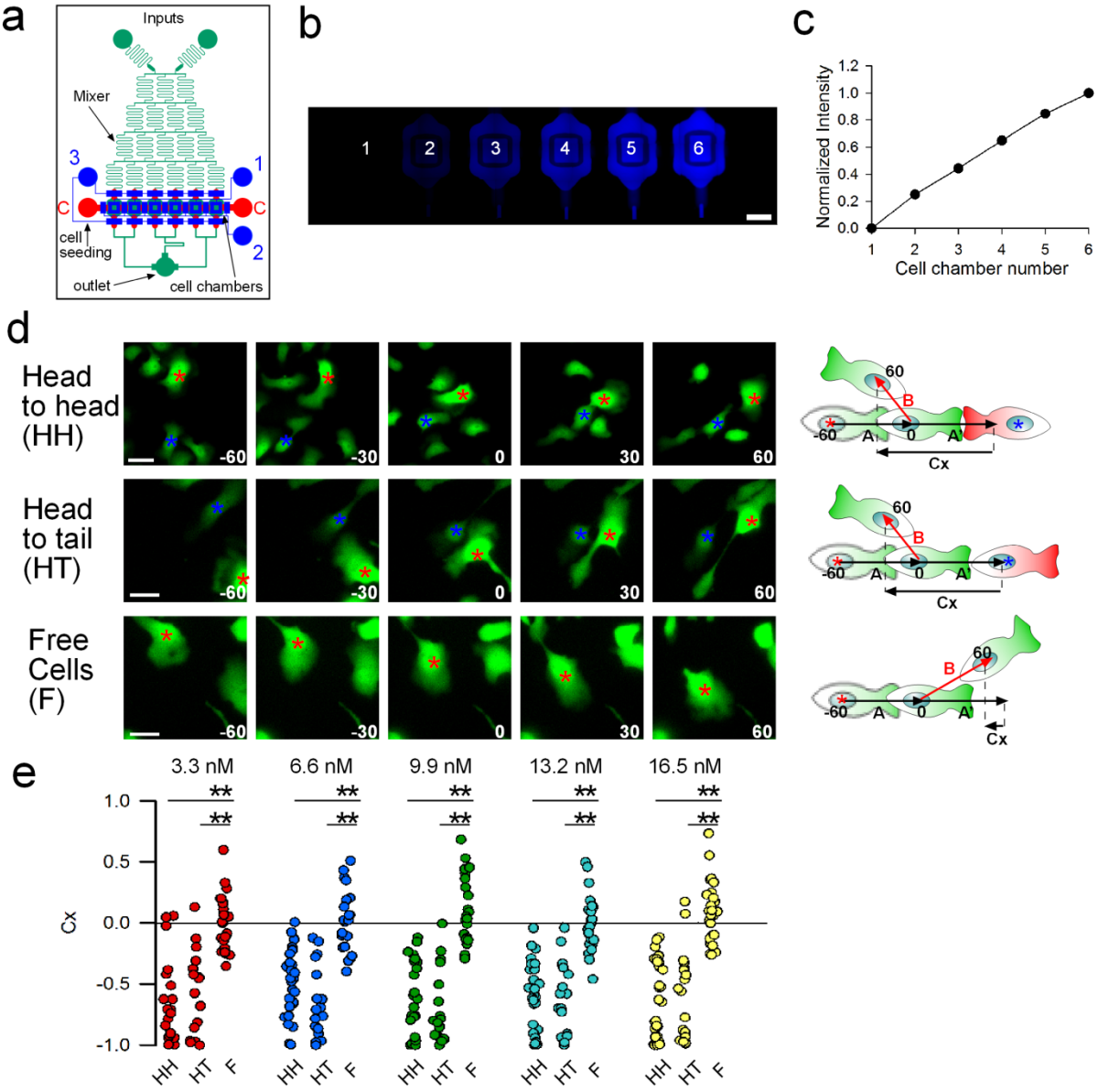

**Supplementary figure 3. Characterization of chemotaxis and CIL in MTLn3 cells.** (**a**) MTLn3 cells in microchannels without EGF. (**b**) MTLn3 cells directionally migrating towards a 2.5-5.8 nM gradient of EGF. (**c**) MTLn3 cells chemotaxis in a 5.0-11.6 nM EGF gradient. In **a-c,** green color denotes GFP expression, the blue color visualizes the gradient of EGF using dextran dye as a proxy, and colored asterisks track individual cell positions. Times are in minutes. Scale bars, 20 μm. (**d-e**) Quantification of the chemotaxis index and velocity of MTLn3 cells in control and different EGF gradients. Data represents the mean from the number of cells indicated from n = 3 independent experiments with error bars showing SEM. (**f-g**) HH (**f**) and HT(**g**) collision outcomes in MTLn3 cells in uniform vs. a gradient of EGF at the indicated concentrations. Statistical comparisons are made between the same outcome (red bars) in uniform vs. gradient. The asterisk denotes a statistically significant difference using a two sided student's t-test. In (**f-g**), number of total collisions are indicated and data represents the mean from n =3 independent experiments with error bars indicating SEM. * = $p < .05$.

# Supplementary figure 4



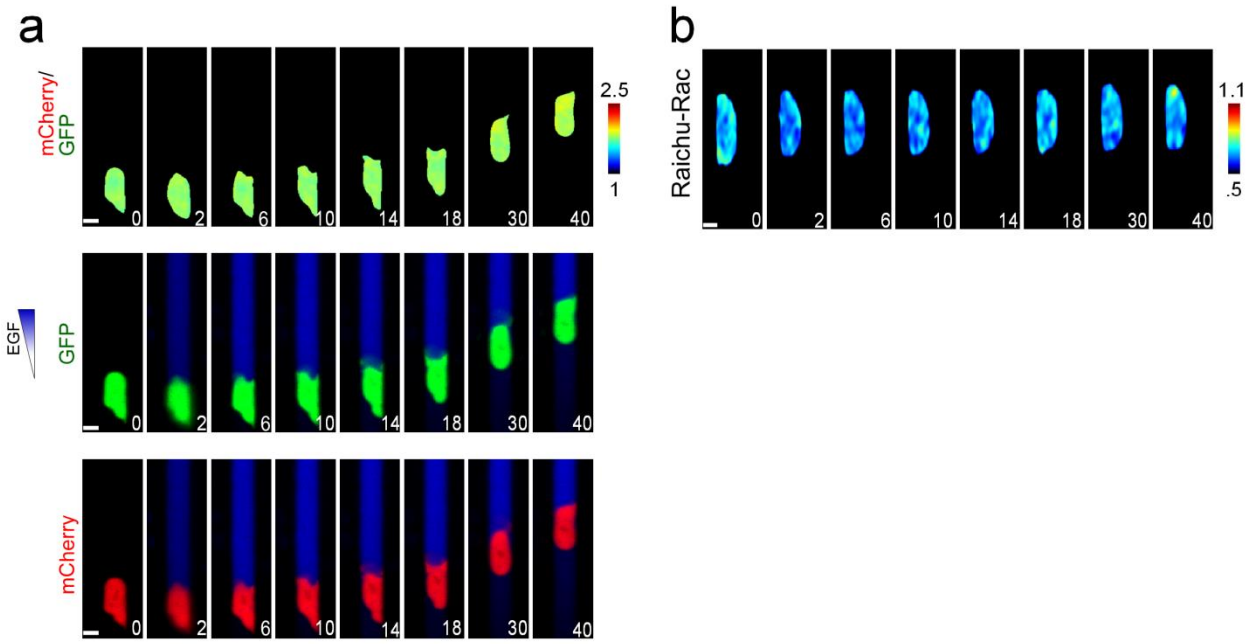

**Supplementary Figure 4. Characterization of CIL in uniform and gradients of EGF.** (**a**) Histograms of the CIL response time (stop in migration, protrusion collapse, and protrusion reformation) after initial contact in head to head (HH) collisions in different uniform concentrations of EGF. Data is collected from 3 independent experiments per condition. (**b-c**) Characterization of protrusions in cells which do not undergo CIL in HH collision outcome 1 (**b**) and HH collision outcome 3 (**c**) in uniform EGF. Cartoons on left illustrate how cells which do not experience CIL (blue cell) either collapse and reform their protrusion in the same direction or maintain their protrusion.Data is accumulated from 3 independent experiments. (**d**) Histograms of the CIL response time in head to head (HH) collisions in different gradients of EGF. Data is collected from 3 independent experiments per condition. (**e-f**) Characterization of protrusions in cells which do not undergo CIL in HH collision outcome 1 (**e**) and HH collision outcome 3 (**f**) in gradients of EGF. Data is accumulated from 3 independent experiments.

# Supplementary figure 5



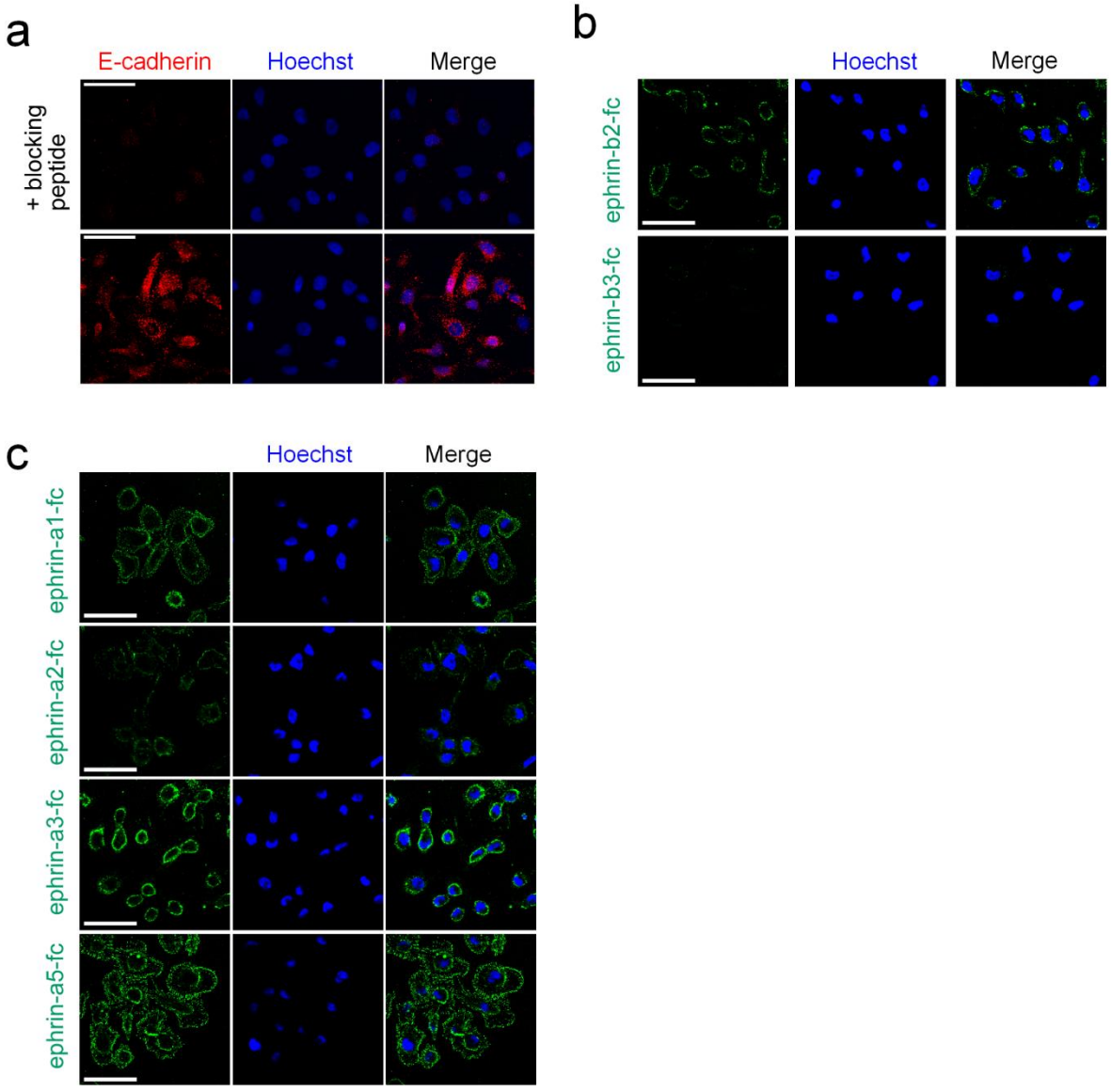

**Supplementary Figure 5. CIL occurs in an EGF dose independent manner in 2D.** (**a**) The schematic of the microfluidic device used to produce multiple doses of EGF in a single experiment. The green gradient generation and cell chamber layer is 100 μm and the red rounded fluid layer is 30 μm. The blue layer (40 μm) is a control valve layer modulating flow in the red layer. (**b**) Sample image of different doses of EGF produced in the numbered cell chambers visualized with a dextran dye. (**c**) Quantification of mean fluorescence intensity in the labeled cell chambers in (**b**), showing the different doses produced. (**d**) MTLn3-B1 cells undergoing head to head (HH) (top panel) or head to tail (HT) (middle panel) collisions as opposed to free movement (**F**) (bottom panel). Corresponding metrics used to analyze them are shown on the side. The vector, Cx, represents the change in acceleration due to either a collision or random migration. Cx is normalized to account for differences in velocity. Green color indicates GFP expression. Colored asterisks track individual cells. Times are in minutes. Scale bars, 20 μm. (e) Quantification of Cx in HH and HT collisions vs. free moving cells (F) in different doses of EGF. n > = 15 cells from 3 independent experiments per condition. ** = p <1e-3 from a Mann-Whitney rank sum test.

# Supplementary figure 6



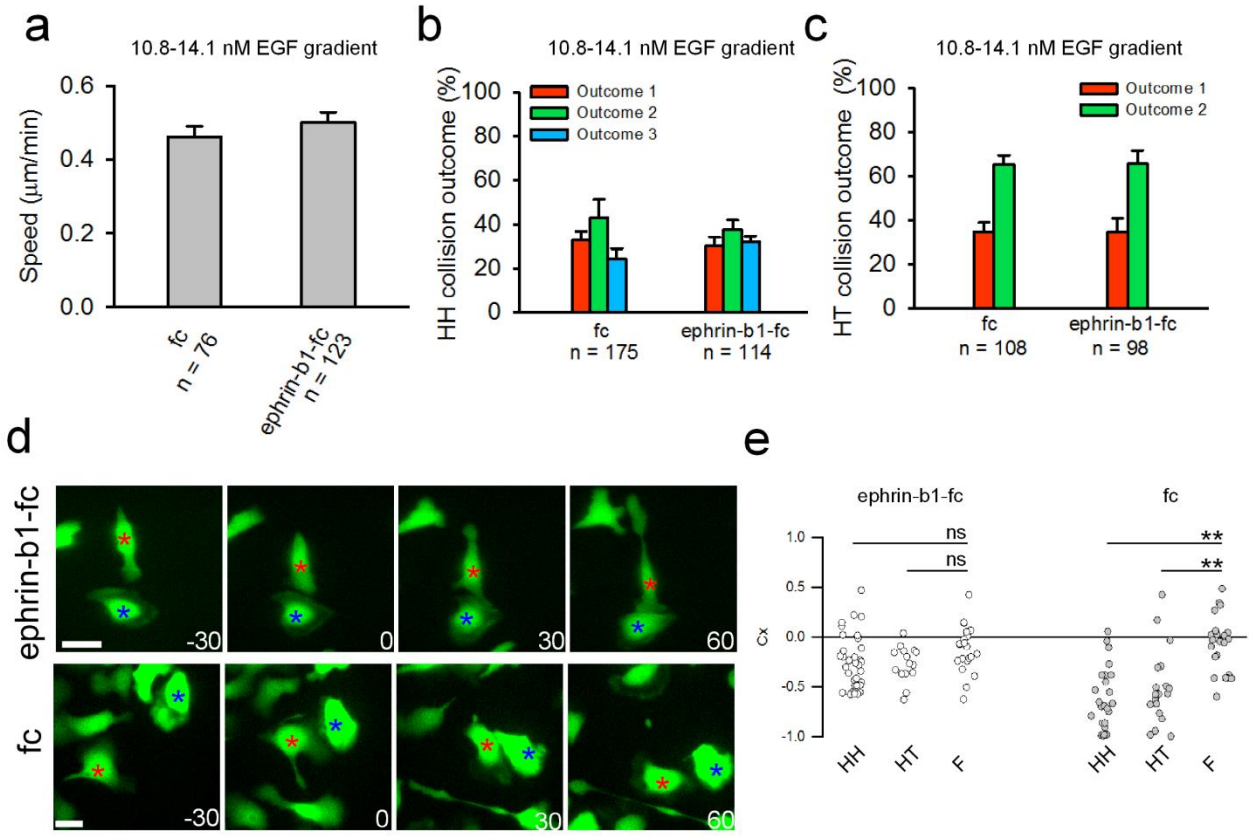

**Supplementary Figure 6**. **PI3K and Rac activity controls.** (**a**) A representative time series of a MTLn3-B1 cell expressing GFP and mCherry chemotaxing to a gradient of EGF (2.5-5.8 nM). The pseudocolor in the top series of images represents the ratio between mCherry and GFP. Green color denotes GFP expression, red color denotes mCherry expression, and the blue color visualizes the EGF gradient via a dextran dye. Quantification of control data is found in **Figure 4b**. (**b**) Representative time series of a MTLn3-B1 cell expressing a Raichu-Rac FRET sensor in a microchannel without EGF. Cells do not polarize and remain stationary. The pseudocolor represents the FRET ratio (YFP FRET/CFP). Quantification of control data is found in **Figure 4d**. Times are in minutes. Scales bars, 10 μm.

# Supplementary figure 7



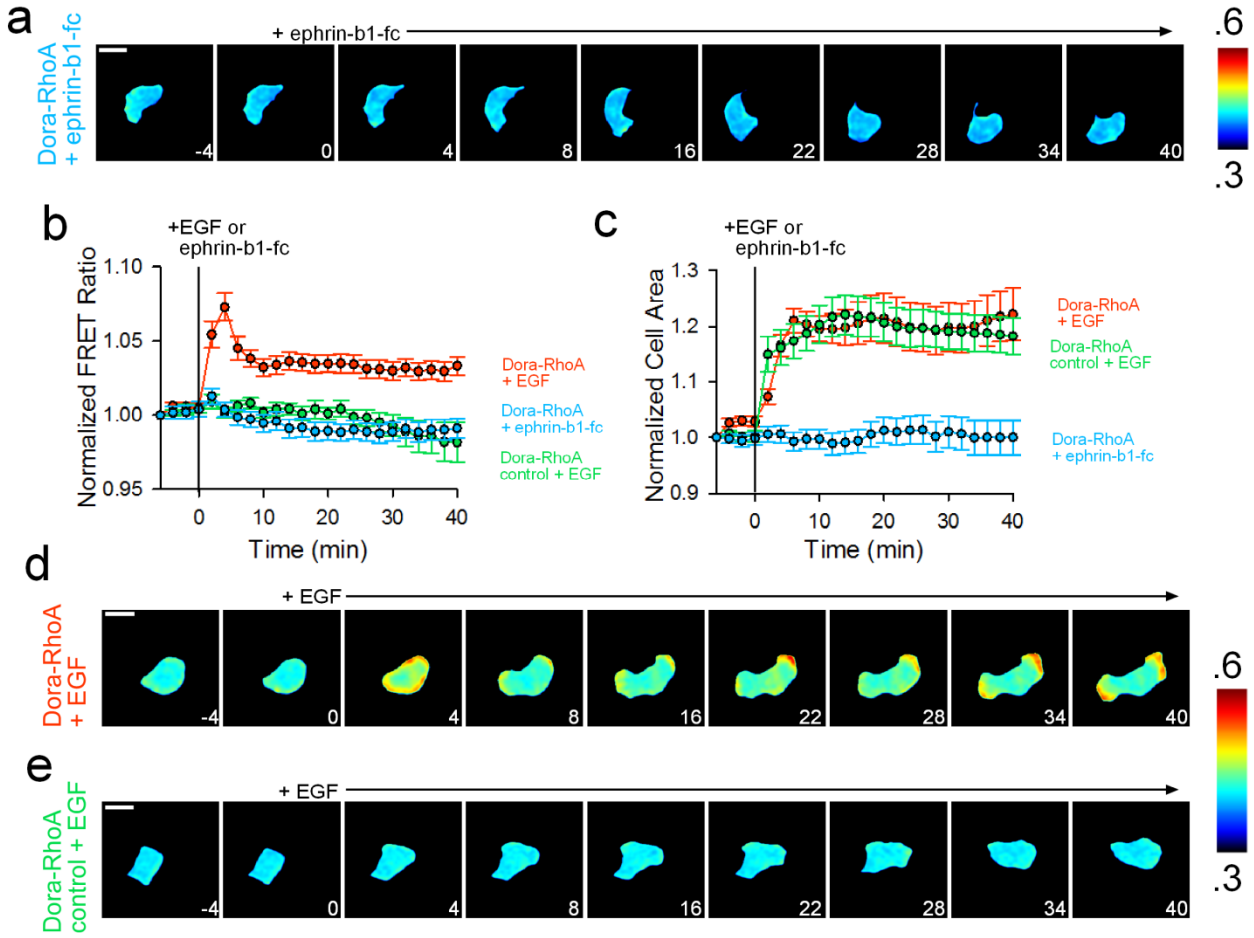

**Supplementary Figure 7**. **Immunostaining of E-cadherin and the binding of various ephrins**. (**a**) Representative images of E-cadherin immunostaining shown in red with and without a blocking peptide in MTLn3-B1 cells. Hoechst 33342 staining shown in blue denotes nuclei. (**b-c**) Representative images of the binding of the indicated ephrin proteins to MTLn3-B1 cells shown in green by immunostaining against the fc domain. Hoechst 33342 staining in blue denotes nuclei. Scale bars, 50 μm.

# Supplementary figure 8



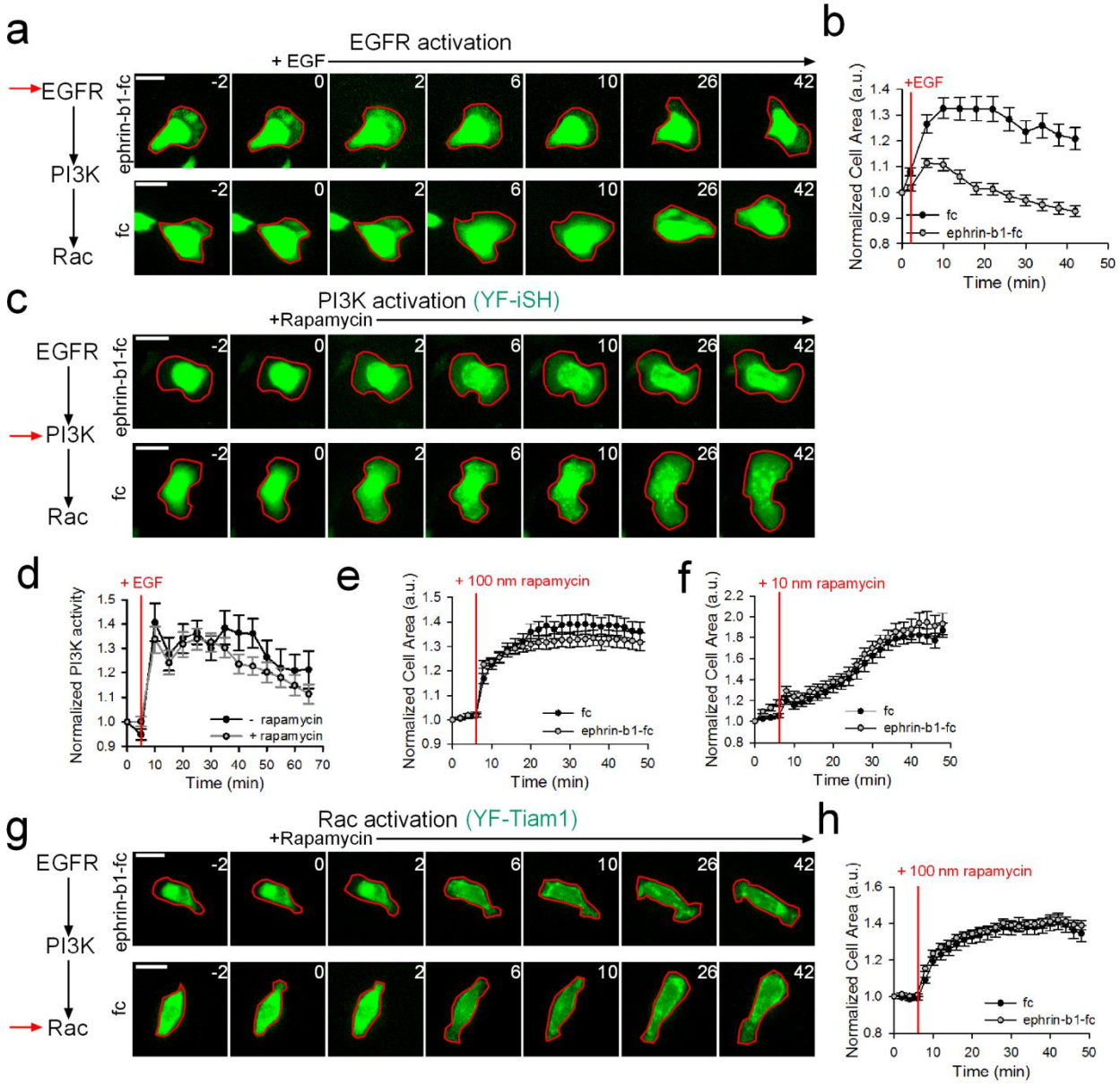

**Supplementary Figure 8. Additional experiments with unclustered ephrin-b1-fc and fc in 1D and 2D.** (**a**) Quantification of MTLn3-B1 cell speed in the presence of 4 μg/ml unclustered ephrin-b1-fc or fc. Collision data is presented in Fig. 6f,g. (**b,c**) HH (**b**) and HT(**c**) collision outcomes in MTLn3-B1 cells in 10.8-14.1 nM EGF gradients after pretreatment with 1 μg/ml ephrin-b1-fc or fc. Number of total cells are indicated and data represents the mean from n =3 independent experiments with error bars indicating SEM. (**d**) Representative images of MTLn3 B1 cells undergoing CIL in presence of 4 μg/ml unclustered ephrin-b1-fc or fc.Times are in minutes. Scale bars, 20 μm. (**e**) Quantification of Cx, which represents the change in acceleration due to either a collision or random migration, in head to head (HH) and head to tail (HT) collisions and freely migrating cells. Changes in Cx are no longer significant in HH and HT collisions after incubation with inclustered ephrin-b1-fc ($P > .05$) as opposed to incubation with unclustered fc, as assessed with a Mann-Whitney Rank Sum test. ** = $p<1e-2$. n>= 15 cells from 3 independent experiments per conditions.

# Supplementary figure 9



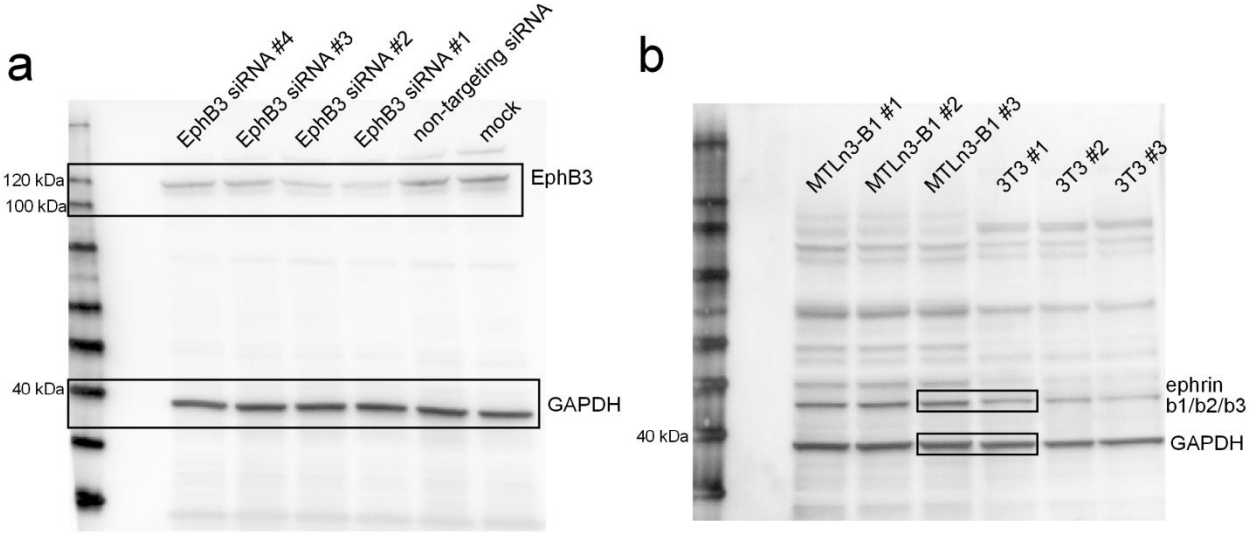

**Supplementary Figure 9. RhoA is not activated by EphB signaling.** (**a**) Representative images of RhoA activity from a Dora-Rhoa FRET construct in MTLn3-B1 cells during clustered ephrin-b1-fc stimulation. (**b-c**) Quantification of the FRET ratio (b) and cell area (c) in the conditions specified. Data indicates the mean from n >= 26 cells per condition from n = 3 independent experiments with error bars representing SEM.(**d-e**) Representative images of RhoA activity from a Dora-RhoA FRET(**d**) or control Dora-Rhoa FRET(**e**) constructs in MTLn3-B1 cells during EGF stimulation. Activites are shown in pseudocolor. Times are in minutes. Scale bars, 20 μm.

# Supplementary figure 10




**Supplementary Figure 10. EphB mediated inhibition of EGF signaling occurs upstream of PI3K.** In all experiments, MTLn3-B1 cells are pretreated with either clustered ephrin-b1-fc or fc (**a-c**,**e-h**) or rapamycin (**d**) for 45 minutes before indicated treatment. (**a**) Representative images of MTLn3-B1 cells responding to 50ng/ml EGF with either ephrin-b1-fc or fc pretreatment. Red outlines trace cell boundaries. Times are in minutes. Scale bars, 20 μm. (**b**) Quantification of cell area after EGF stimulation from (**a**). Data is the mean from n >= 80 cells per conditions from n = 3 independent experiments. (**c**,**g**) Representative images of direct (**c**) PI3K or (**e**) Rac activation after the addtion of 100 nm rapamycin with either ephrin-b1-fc or fc pretreatment. Red outlines trace cell area. Times are in minutes. Scale bars, 20 μm. (**d**) Quantification of PI3K activity with and without 100 nm rapamycin pretreatment after 50 ng/ml EGF stimulation. n >= 17 cells per condition from 3 independent experiments. (**e**,**f**,**h**) Quantification of cell area after activation of PI3K(**e**,**f**) or Rac (**h**) with 100 nm rapamycin (**e**,**h**) or 10 nm rapamycin (**f**) after indicated pretreatments. Data is the mean from n >= 30 cells per condition from n = 3 independent experiments. Error bars represent SEM in all plots.

# Supplementary Figure 11




**Supplementary Figure 11. Uncropped Western blots.** (**a**) Uncropped western blot from Fig. 5g. Black box indicates cropped region used for the figure. (**b**) Uncropped western blot from Fig. 5j. Black box indicates cropped region used for the figure.